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(54) Title: LIVE ATTENUATED ALDOLASE-NEGATIVE BACTERIAL VACCINE

(57) Abstract: The present invention relates to live attenuated bacteria for use in a medicament. The invention also relates to vaccines based upon such bacteria useful for the prevention of microbial pathogenesis and to the use of such bacteria for the manufacture of vaccine. Finally, the invention relates to methods for the preparation of such vaccines.

Live attenuated aldolase-negative bacterial vaccine

The present invention relates to live attenuated bacteria for use in a medicament, to vaccines based upon such bacteria useful for the prevention of microbial pathogenesis, to the
5 use of such bacteria for the manufacture of a vaccine and to methods for the preparation of such vaccines.

Immunity to microbial pathogenesis is one means by which a warm blooded animal avoids pathogenesis, or suffers a less intense pathogenic state. Incomplete immunity to a given
10 pathogen results in morbidity and mortality in a population exposed to a pathogen. It is generally agreed that vaccines based on live but attenuated micro-organisms (live attenuated vaccines) induce a highly effective type of immune response. Such vaccines have the advantage that, once the animal host has been vaccinated, entry of the microbial pathogen into the host induces an accelerated recall of earlier, cell-mediated or humoral immunity which is
15 able to control further growth of the organism before the infection can assume clinically significant proportions. Vaccines based on a killed pathogen (killed vaccine) are generally conceded to be unable to achieve this type of response. However, vaccines that contain a live pathogen present, depending on the level of attenuation, the danger that the vaccinated host upon vaccination may contract the disease against which protection is being sought.

20 Vaccines against bacteria belonging to e.g. the closely related families of Escherichia and Salmonella follow the general rules given above. Many members of these families of bacteria are pathogenic due to the fact that they infect the digestive tract and/or the bladder.
The pathogenic effect of these bacteria is closely related to their ability to colonise the mucosal
25 layers of the digestive tract and/or the bladder. It is the phenomenon of colonisation that leads to the prolonged presence of the pathogen in the digestive tract and to a very close contact of the pathogen to the mucosal layers, which can also lead to invasion of other tissues. Thus, at the same time, paradoxically, it's due to the fact that these bacteria colonise the digestive tract and/or the bladder, and thus at the same time cause disease, that the immune system is
30 triggered to develop a certain level of immune response. Consequently, this immune response is developed too late to suppress the pathogenic effect of the colonising bacteria.

It would be desirable to have a live attenuated vaccine against enteric infections, that possesses the immunising attributes of a live micro-organism but that is not capable of causing undesirable side effects upon vaccination.

A first prerequisite for such a vaccine would seem to be its ability to colonise the digestive tract.

5 It is the phenomenon of colonisation that provides a firm trigger of the immune system.

It is an objective of the present invention to provide a live attenuated bacterium that does provide protection against infection with wild-type bacteria.

10 Surprisingly it was found now that bacterial strains of the genus *Escherichia*, *Salmonella*, and *Yersinia* having a mutation in the *eda* gene that prevents the synthesis of functional Eda protein are very well capable of inducing a protective immune response against virulent wild-type bacteria in the host animal. Such mutants are, first of all, safe when used as live attenuated vaccine strains. Moreover, they are capable of preventing colonisation of wild type strains.

15 Merely as an example, when such vaccines are administered to chickens of over two weeks of age they are capable of inducing immunity even to the level that neither the vaccine strain nor the challenge strain can be re-isolated from the cloaca after challenge.

This is indeed against all expectations, since it has been shown that *E. coli* Eda-negative mutants do not colonise the mouse large intestine (Sweeney, N.J. et al., in Infect. & Immun. 64: 20 3504-3511 (1996)). This is even the case if the mice are previously streptomycin-treated. Such treatment leads to the eradication of all facultative bacteria from the intestines, so that all possible intestinal niches are in principle available. Nevertheless, Eda-negative mutants were shown, contrary to their wild-type counterparts, to be unable to colonise in mice. Thus, Eda-negative mutants were also not expected to colonise the digestive tract of other mammals or poultry, so they consequently were not expected to come into sufficiently close contact with the immune system to trigger it anyway.

Therefore, one embodiment of the present invention relates to a live attenuated bacterium that 30 is not capable to express a functional Eda protein as a result of a mutation in the *eda* gene, for use in a vaccine.

Additionally, Eda-mutants as described in the invention show a very low pathogenicity, which makes them attractive as vaccine strains.

The *eda* gene, encoding the Eda protein plays a key role in what is known as the Entner-

5 Doudoroff Pathway. Its gene product, the Eda-protein is an enzyme, also known as KDPG-aldolase. This enzyme is used in the metabolism of glucuronate, galacturonate and gluconate. Fraenkel, D.G. has described the Entner-Doudoroff pathway in e.g. *E.coli* and *Salmonella* at p. 142-150 in F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter and H.E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology.

10 American Society for Microbiology, Washington D.C. (1987).

Egan et al., have analysed the Entner-Doudoroff pathway in *E. coli* and they have analysed the sequence and localized promoters for the *edd-eda* operon (Egan et al., J. Bacteriology 174: 4638-4646 (1992)).

15 The sequence of the DNA encoding Eda of *Salmonella typhimurium* is depicted in SEQ ID NO:

1. The sequence of the Eda protein itself is given in SEQ ID NO: 2.

The fact that *eda* mutants are not expected to colonise the digestive tract explains why, although such mutants were known in the art, they have never been suggested to be potential

20 live attenuated vaccine candidates.

Due to its key position in carbohydrate metabolism, the *eda* gene and its gene product Eda are relatively widespread in the bacterial realm. The Eda protein is a highly conserved protein. It can be found in e.g. *Escherichia coli*, in *Salmonella* species, more specifically *Salmonella*

25 *enterica* species, such as serotype Typhimurium, Enteritidis and Dublin and in *Yersinia* species such as *Y. pestis*.

The mutation can be an insertion, a deletion, a substitution or a combination thereof, provided that the mutation leads to the failure to express a functional Eda protein. A functional Eda

30 protein is understood to be a protein having the regulating characteristics of the wild-type protein. Thus, an Eda protein that is defective in at least one of its functions is considered to be a non-functional Eda protein. More specifically, a non-functional Eda protein would not or to a

lesser extend, when compared to its wild-type counterpart, be able to mediate the synthesis of KDPG from pyruvate and glyceraldehyde-3-phosphate and vice versa. As a result, a strain having a non-functional Eda-protein would not or to a lesser extent, when compared to its wild-type counterpart, be able to utilise glucuronate, galacturonate and gluconate.

5

Live attenuated bacteria for use according to the invention can be obtained in several ways. One possible way of obtaining such bacteria is by means of classical methods such as the treatment of wild-type bacteria having the *eda* gene with mutagenic agents such as base analogues, treatment with ultraviolet light or temperature treatment.

10

Strains that do not produce a functional Eda protein can easily be picked up. Such mutants can not utilise glucuronate, galacturonate or gluconate, but they can utilise glucose and galactonate.

They can therefore, on the basis of these specific abilities, very easily be selected *in vitro*. A 15 detailed description of how to make such a selection has been described by Sweeney, N.J. et al., in Infect. & Immun. 64: 3504-3511 (1996).

The nature of a mutation if caused by classical mutation techniques is unknown. This may be a point mutation which may, although this is unlikely to happen, eventually revert to wild-type. In 20 order to avoid this small risk, transposon mutagenesis would be a good alternative.

Mutagenesis by transposon mutagenesis, is also a mutagenesis-technique well-known in the art. This is a mutation accomplished at a localised site in the chromosome. Transposon- insertions can not be targeted to a specific gene. It is however very easy to pick up *eda*- mutants since they do not grow *in vitro* without nutrient compensation for lack of Eda activity.

25 Therefore, they can easily be selected from a pool of randomly transposon-mutated bacteria.

A much more attractive way of making mutations, i.e. the introduction of a mutation at a predetermined site, rather deliberately than randomly, is offered by recombinant DNA- technology. Such a mutation may again be an insertion, a deletion, a replacement of one 30 nucleotide by another one or a combination thereof, with the only proviso that the mutated gene no longer encodes functional Eda. Such a mutation can e.g. be made by deletion of a number of base pairs. Even very small deletions such a stretches of 10 base pairs can already render

Eda non-functional. Even the deletion of one single base pair may already lead to a non-functional Eda, since as a result of such a mutation, the other base pairs are no longer in the correct reading frame. Each deletion or insertion of a number of base pairs indivisible by three causes such a frame shift. More preferably, a longer stretch is removed e.g. 100 base pairs.

5 Even more preferably, the whole *eda* gene is deleted.

It can easily be seen, that especially mutations introducing a stop-codon in the open reading frame, or mutations causing a frame-shift in the open reading frame are very suitable to obtain a strain which no longer encodes functional Eda.

All techniques for the construction of Eda-negative mutants are well-known standard

10 techniques. They relate to cloning of the Eda-gene, modification of the gene sequence by site-directed mutagenesis, restriction enzyme digestion followed by re-ligation or PCR-approaches and to subsequent replacement of the wild type *eda* gene with the mutant gene (allelic exchange or allelic replacement). Standard recombinant DNA techniques such as cloning the *eda* gene in a plasmid, digestion of the gene with a restriction enzyme, followed by
15 endonuclease treatment, re-ligation and homologous recombination in the host strain, are all known in the art and described i.a. in Maniatis/Sambrook (Sambrook, J. et al. Molecular cloning: a laboratory manual. ISBN 0-87969-309-6). Site-directed mutations can e.g. be made by means of in vitro site directed mutagenesis using the Transformer® kit sold by Clontech.
PCR-techniques are extensively described in (Dieffenbach & Dreksler; PCR primers, a
20 laboratory manual. ISBN 0-87969-447-3 and ISBN 0-87969-447-5).

The *eda* gene comprises not only the coding sequence encoding the Eda protein, but also regulatory sequences such as the promoter. The gene also comprises sites essential for correct translation of the Eda mRNA, such as the ribosome binding site.

25 Therefore, not only mutations in the coding regions but also mutations in those sequences essential for correct transcription and translation are considered to fall within the scope of the invention.

In a preferred embodiment, the invention relates to live attenuated bacteria of the genera

30 *Escherichia*, *Salmonella* and *Yersinia* for use in a vaccine.

In a more preferred form of the invention, the live attenuated bacterium according to the invention is selected from the group consisting of *S. enterica* serotype *typhimurium*, *enteritidis*, *choleraesuis*, *dublin*, *typhi*, *gallinarum*, *abortusovis*, *abortus-equi*, *pullorum*, *E. coli* or *Y. pestis*. These bacterial genera comprise a large number of species that are pathogenic to both
5 humans and a variety of different animals.

In an even more preferred form thereof, the live attenuated bacterium according to the invention is selected from the group consisting of *S. enterica* serotype *typhimurium*, *enteritidis*, *gallinarum*, *pullorum*, *E. coli* or *Y. pestis*.

10 Well-defined and deliberately made mutations involving the deletion of fragments of the *eda* gene or even the whole gene or the insertion of heterologous DNA-fragments or both, have the advantage, in comparison to classically induced mutations, that they will not revert to the wild-type situation.
15 Thus, in an even more preferred form, this embodiment of the invention refers to live attenuated bacteria in which the *eda* gene comprises an insertion and/or a deletion.

Given the large amount of vaccines given nowadays to both pets and farm animals, it is clear that combined administration of several vaccines would be desirable, if only for reasons of
20 decreased vaccination costs. It is therefore very attractive to use live attenuated bacteria as a recombinant carrier for heterologous genes, encoding antigens selected from other pathogenic micro-organisms or viruses. Administration of such a recombinant carrier has the advantage that immunity is induced against two or more diseases at the same time. The live attenuated bacteria for use in a vaccine, according to the present invention provide very suitable carriers
25 for heterologous genes, since the gene encoding the Eda protein can be used as an insertion site for such heterologous genes. The use of the *eda* gene as an insertion site has the advantage that at the same time the *eda* gene is inactivated and the newly introduced heterologous gene can be expressed (in concert with the homologous bacterial genes). The construction of such recombinant carriers can be done routinely, using standard molecular
30 biology techniques such as allelic exchange.

Therefore, another embodiment of the invention relates to live attenuated recombinant bacteria, preferably of the genera *Escherichia*, *Salmonella* and *Yersinia* that do not produce a functional Eda protein and in which a heterologous gene is inserted, for use in a vaccine. Such a heterologous gene can, as mentioned above, e.g. be a gene encoding an antigen selected from other pathogenic micro-organisms or viruses. Such genes can e.g. be derived from pathogenic herpesviruses (e.g. the genes encoding the structural proteins of herpesviruses), retroviruses (e.g. the gp160 envelope protein), adenoviruses and the like.

5 Also a heterologous gene can be obtained from pathogenic bacteria. As an example, genes encoding bacterial toxins such as *Actinobacillus pleuropneumoniae* toxins, *Clostridium* toxins, outer membrane proteins and the like are very suitable bacterial heterologous genes.

10 Another possibility is to insert a gene encoding a protein involved in triggering the immune system, such as an interleukin or an interferon, or another gene involved in immune-regulation.

15 Insertion of the heterologous gene in the *eda* gene is advantageous, since in that case there is no need to find a new suitable insertion site for the heterologous gene, and at the same time the *eda* gene is knocked out.

Thus, in a preferred form of this embodiment the heterologous gene is inserted in the *eda* gene. The heterologous gene can be inserted somewhere in the *eda* gene or it can be inserted at the site of the *eda* gene while this gene has been partially or completely deleted.

20 Because of their unexpected attenuated but immunogenic character *in vivo*, the bacteria for use in a vaccine, according to the invention are very suitable as a basis for live attenuated vaccines. Thus, still another embodiment of the invention relates to live attenuated vaccines for the protection of animals and humans against infection with a bacterium of which the wild type form 25 comprises a *eda* gene.

Such vaccines comprise an immunogenically effective amount of a live attenuated bacterium for use in a vaccine, according to the invention or a live recombinant carrier bacterium according to the invention, and a pharmaceutically acceptable carrier.

30 Preferably, the vaccine comprises a live attenuated bacterium according to the invention, selected from the group of *Escherichia*, *Salmonella* and *Yersinia*.

Immunogenically effective means that the amount of live attenuated bacteria administered at vaccination is sufficient to induce in the host an effective immune response against virulent forms of the bacterium.

In addition to an immunogenically effective amount of the live attenuated bacterium described above, a vaccine according to the present invention also contains a pharmaceutically acceptable carrier. Such a carrier may be as simple as water, but it may e.g. also comprise culture fluid in which the bacteria were cultured. Another suitable carrier is e.g. a solution of physiological salt concentration.

10 The useful dosage to be administered will vary depending on the age, weight and animal vaccinated, the mode of administration and the type of pathogen against which vaccination is sought.

The vaccine may comprise any dose of bacteria, sufficient to evoke an immune response.

15 Doses ranging between 10^3 and 10^{10} bacteria are e.g. very suitable doses.

Optionally, one or more compounds having adjuvant activity may be added to the vaccine.

Adjuvants are non-specific stimulators of the immune system. They enhance the immune response of the host to the vaccine. Examples of adjuvants known in the art are Freunds

20 Complete and Incomplete adjuvant, vitamin E, non-ionic block polymers, muramyldipeptides, ISCOMs (immune stimulating complexes, cf. for instance European Patent EP 109942), Saponins, mineral oil, vegetable oil, and Carbopol.

Adjuvants, specially suitable for mucosal application are e.g. the *E. coli* heat-labile toxin (LT) or Cholera toxin (CT).

25 Other suitable adjuvants are for example aluminium hydroxide, aluminium phosphate or aluminium oxide, oil-emulsions (e.g. of Bayol F (R) or Marcol 52 (R)), saponins or vitamin-E solubilisate.

Therefore, in a preferred form, the vaccines according to the present invention comprise an
30 adjuvant.

Other examples of pharmaceutically acceptable carriers or diluents useful in the present invention include stabilisers such as SPGA, carbohydrates (e.g. sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as albumin or casein, protein containing agents such as bovine serum or skimmed milk and buffers (e.g. phosphate buffer).

5 Especially when such stabilisers are added to the vaccine, the vaccine is very suitable for freeze-drying. Therefore, in a more preferred form, the vaccine is in a freeze-dried form.

For administration to animals or humans, the vaccine according to the present invention can be given inter alia intranasally, intradermally, subcutaneously, orally, by aerosol or intramuscularly.

10 For application to poultry, wing web and eye-drop administration are very suitable. The skilled person would know how to administer a vaccine according to the invention, because the method would most likely not differ from the methods followed for vaccination with presently existing bacterial vaccines. A vaccine according to the invention, especially when it comprises bacteria belonging to the family of *E. coli*, *Salmonella* or *Yersinia* would preferably be given
15 orally.

Still another embodiment relates to the use of a bacterium or a recombinant bacterium according to the invention for the manufacture of a vaccine for the protection of animals and humans against infection with a wild type bacterium or the pathogenic effects of infection.

20 Still another embodiment of the invention relates to methods for the preparation of a vaccine according to the invention. Such methods comprise the admixing of a live attenuated bacterium according to the invention or a live recombinant carrier bacterium according to the invention, and a pharmaceutically acceptable carrier.

25

EXAMPLES**Example 1****Construction of Eda-negative mutant.**

The *eda* deletions were made using a modified Wanner method (PNAS June 6, 2000).

5 97(12):6640-45). Primers to the 5' and 3' *eda* flanking sequences of *S. enteritidis* SE5609 were designed with *PstI* sites (*PstI* *eda* 5': cta gct gca ggt gct aag cgg taa tct ggg and *PstI* *eda* 3': cta gct gca gaa gag att gct cgt cat gtg g) and the PCR product was cloned into pBluescript SKII+ (pBSeda). *BglII* containing primers, to the *eda* promoter region (ctag aga tct ctgcctgattacta gtgtg) and the 3' end (ctag agatct aag ccg ttaaatgccccatgg) were used to reverse PCR amplify
10 the flanking and vector sequences from pBSeda. *BglII* digestion and subsequent ligation created an *eda* deletion clone called pBSedaΔ. A 1.2 kb *BamHI* digested chloramphenicol resistant gene was inserted into the *BglII* site of pBSedaΔ to produce pBSedaΔcam.
15 pBSedaΔcam was digested with *PstI* and the insert was separated from vector sequences and used as the template for PCR. Eight 100 µl reactions were pooled, 5 µl was checked on a gel, and the linear PCR product was ethanol precipitated and resuspended in 2-4 µl of water.

S. enteritidis SE5609 cells were electroporated with the temperature sensitive plasmid pKD46. When these cells are grown at 30°C in the presence of arabinose the plasmid expresses the lambda Red recombinase. The cells ($A_{600}=0.6$) were made competent for electroporation by
20 centrifugation and washing 3-4X with cold 10% glycerol. The linear PCR product was then electroporated into the competent cells. The recombinase acts to replace the wild type *eda* gene with the deleted *eda* gene containing the chloramphenicol cassette. SE5609 clones containing the deleted *eda* gene containing the chloramphenicol cassette were selected by overnight growth at 37°C on Luria agar plates containing chloramphenicol (30 µg/ml), which
25 also resulted in loss of the temperature sensitive pKD46 plasmid.

A different set of flanking *eda* 5' (ctagctgca gcc tca tat tcc gga cct gag c) and *eda* 3' (cta gct gca ggt gac ggt aaa agg cta atg cg) primers were used to verify one of the SE5609-*eda*⁽⁻⁾ *eda* mutants. An amplified fragment containing the wild type SE5609 *eda* gene produced a 927bp
30 band as expected, while the *eda* deletion/chloramphenicol mutant gave the expected 1677bp product. 1.8mM MgCl₂, with Finnzyme DyNAzyme[®] polymerase was used in the reaction. Cycling conditions were 1X 94°C 4min; 30X 94°C 15sec, 55°C 30sec, 72°C 130sec; 1X 72°C

7min. In addition, the same SE5609-eda⁽⁺⁾ eda mutant was also confirmed by its inability to grow in liquid M9 minimal medium containing 0.2% (w/w) gluconate as the sole carbon source.

5 **Example 2**

Safety, vaccination- and challenge-tests.

Experimental design

To test both safety and efficacy, chickens were orally inoculated at 6 and 14 weeks of age with respectively 1.1×10^8 CFU and 4.1×10^8 CFU of SE5609-eda⁽⁺⁾.

10 Safety was assessed by clinical observation after vaccination. Also, cloaca swabs were taken at days 7 and 14 after each vaccination to determine the presence of the vaccine strain in the intestinal tract. Swabs were used to inoculate Brilliant Green Agars (BGA) directly and after enrichment in Rappaport Vassiliades Broth.

To test for efficacy, the vaccinated chickens and unvaccinated controls received an oral 15 challenge infection with 1.3×10^8 CFU of a naladixic acid resistant wild-type S.e. strain at 16 weeks of age. Cloaca swabs were taken 3, 7 and 14 days after challenge to determine the rate of colonization by the challenge strain. Swabs were inoculated on BGA containing naladixic acid (BGAnal) directly and also after incubation in an enrichment medium (buffered peptone water containing nal).

20

Animals

Commercial laying hens were obtained from a Salmonella free flock.

Results

25 No clinical abnormalities were observed after both oral vaccinations.

First of all, the SE5609-eda⁽⁺⁾ strain was not cultured from cloacal swabs of the vaccinated animals on days 7 and 14, indicating that the strain was impaired in its ability to colonize the digestive tract of chickens.

Moreover, as shown in Table 1, vaccination with SE5609-eda⁽⁺⁾ resulted in a very significant 30 reduction in colonization of the digestive tract by the challenge strain.

Table 1 Reisolation of the challenge strain from cloaca swabs

	S.e. (nal ^r) positive	
Day post challenge	SE5609-eda	Control
3	1/15 ^a	11/15
7	1/15 ^a	9/14
14	0/15	2/14

^a: significantly different from control (p<0.01, Fisher exact test)

Conclusion: a vaccine based upon a *Salmonella enteritidis* eda⁽⁺⁾ strain which is a wild-type strain having eda⁽⁺⁾ as a single attenuation is safe. Moreover, vaccination with this *Salmonella enteritidis* eda⁽⁺⁾ strain resulted in complete prevention of colonization of the digestive tract by both the vaccine strain and the challenge strain.

Claims

- 1) Live attenuated bacterium of the genus *Escherichia*, *Salmonella* or *Yersinia*, said bacterium not being capable to express a functional Eda protein as a result of a mutation in the *eda* gene,
5 for use in a vaccine.
- 2) Live attenuated bacterium for use according to claim 1, wherein said bacterium is selected from the group consisting of *E. coli*, *Y. pestis*, *S. enterica* serotype *typhimurium*, *enteritidis*, *choleraesuis*, *dublin*, *typhi*, *gallinarum*, *abortusovis*, *abortus-equi*, *pullorum*.
- 10 3) Live attenuated bacterium for use according to claim 1 or 2, characterised in that the mutation comprises an insertion and/or a deletion.
- 15 4) Live attenuated bacterium for use according to claims 1-3, characterised in that said bacterium carries a heterologous gene.
- 5) Live attenuated bacterium for use according to claim 4, characterised in that said heterologous gene is inserted in the *eda* gene
- 20 6) Live attenuated vaccine for the protection of animals or humans against infection with a pathogenic bacterium or the pathogenic effects thereof, characterised in that said vaccine comprises a bacterium as defined in claims 1-5 and a pharmaceutically acceptable carrier.
- 7) Live attenuated vaccine according to claim 6, characterised in that it comprises an adjuvant.
- 25 8) Live attenuated vaccine according to claim 6 or 7, characterised in that it is in a freeze-dried form.
- 9) Use of a live attenuated bacterium as defined in claims 1-5 for the manufacture of a vaccine
30 for the protection of animals against infection with a pathogenic bacterium or the pathogenic effects of infection.

14

10) Method for the preparation of a vaccine according to claims 6-8, characterised in that said method comprises the admixing of a live attenuated bacterium as defined in claims 1-5 and a pharmaceutically acceptable carrier.

5

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, CHEM ABS Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 1 074 266 A (AKZO NOBEL N.V; THE BOARD OF GOVERNORS FOR HIGHER EDUCATION, STATE OF) 7 February 2001 (2001-02-07) page 1, paragraph 1 page 1, paragraph 5 – page 2, paragraph 10 claims	1-10
Y	SWEENEY N J ET AL: "ESCHERICHIA COLI F-18 AND E. COLI K-12 EDA MUTANTS DO NOT COLONIZE THE STREPTOMYCIN-TREATED MOUSE LARGE INTESTINE" INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON, US, vol. 64, no. 9, September 1996 (1996-09), pages 3504-3511, XP000905643 ISSN: 0019-9567 cited in the application the whole document	1-10
	-/-	

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

Inter	Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATSENKO KIRILL A ET AL: "One-step inactivation of chromosomal genes in <i>Escherichia coli</i> K-12 using PCR products" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, US, vol. 97, no. 12, 6 June 2000 (2000-06-06), pages 6640-6645, XP002210218 ISSN: 0027-8424 cited in the application abstract	1-10
A	ALLEN J H ET AL: "A functional cra gene is required for <i>Salmonella enterica</i> serovar <i>Typhimurium</i> virulence in BALB/c mice" INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, US, vol. 68, no. 6, June 2000 (2000-06), pages 3772-3775, XP002229250 ISSN: 0019-9567 the whole document	1-10
A	RAMSEIER T M: "CRA AND THE CONTROL OF CARBON FLUX VIA METABOLIC PATHWAYS" RESEARCH IN MICROBIOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 147, no. 6/7, 1996, pages 489-493, XP001134939 ISSN: 0923-2508 the whole document	1-10

INTERNATIONAL SEARCH REPORT

I	Final application No.
PCT/US2004/027897	

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the International application and necessary to the claimed Invention, the international search was carried out on the basis of:
 - a. type of material
 - a sequence listing
 - table(s) related to the sequence listing
 - b. format of material
 - in written format
 - in computer readable form
 - c. time of filing/furnishing
 - contained in the International application as filed
 - filed together with the International application in computer readable form
 - furnished subsequently to this Authority for the purpose of search
2. In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Inter Application No
PCT/US2004/027897

Patent document cited in search report	Publication date		Patent family member(s)	Publication date
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(54) Title: LIVE ATTENUATED ALDOLASE-NEGATIVE BACTERIAL VACCINE

(57) Abstract: The present invention relates to live attenuated bacteria for use in a medicament. The invention also relates to vaccines based upon such bacteria useful for the prevention of microbial pathogenesis and to the use of such bacteria for the manufacture of vaccine. Finally, the invention relates to methods for the preparation of such vaccines.

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Live attenuated aldolase-negative bacterial vaccine

The present invention relates to live attenuated bacteria for use in a medicament, to vaccines based upon such bacteria useful for the prevention of microbial pathogenesis, to the
5 use of such bacteria for the manufacture of a vaccine and to methods for the preparation of such vaccines.

Immunity to microbial pathogenesis is one means by which a warm blooded animal avoids pathogenesis, or suffers a less intense pathogenic state. Incomplete immunity to a given
10 pathogen results in morbidity and mortality in a population exposed to a pathogen. It is generally agreed that vaccines based on live but attenuated micro-organisms (live attenuated vaccines) induce a highly effective type of immune response. Such vaccines have the advantage that, once the animal host has been vaccinated, entry of the microbial pathogen into the host induces an accelerated recall of earlier, cell-mediated or humoral immunity which is
15 able to control further growth of the organism before the infection can assume clinically significant proportions. Vaccines based on a killed pathogen (killed vaccine) are generally conceded to be unable to achieve this type of response. However, vaccines that contain a live pathogen present, depending on the level of attenuation, the danger that the vaccinated host upon vaccination may contract the disease against which protection is being sought.

20 Vaccines against bacteria belonging to e.g. the closely related families of Escherichia and Salmonella follow the general rules given above. Many members of these families of bacteria are pathogenic due to the fact that they infect the digestive tract and/or the bladder. The pathogenic effect of these bacteria is closely related to their ability to colonise the mucosal
25 layers of the digestive tract and/or the bladder. It is the phenomenon of colonisation that leads to the prolonged presence of the pathogen in the digestive tract and to a very close contact of the pathogen to the mucosal layers, which can also lead to invasion of other tissues. Thus, at the same time, paradoxically, it's due to the fact that these bacteria colonise the digestive tract and/or the bladder, and thus at the same time cause disease, that the immune system is
30 triggered to develop a certain level of immune response. Consequently, this immune response is developed too late to suppress the pathogenic effect of the colonising bacteria.

It would be desirable to have a live attenuated vaccine against enteric infections, that possesses the immunising attributes of a live micro-organism but that is not capable of causing undesirable side effects upon vaccination.

A first prerequisite for such a vaccine would seem to be its ability to colonise the digestive tract.

5 It is the phenomenon of colonisation that provides a firm trigger of the immune system.

It is an objective of the present invention to provide a live attenuated bacterium that does provide protection against infection with wild-type bacteria.

10 Surprisingly it was found now that bacterial strains of the genus *Escherichia*, *Salmonella*, and *Yersinia* having a mutation in the *eda* gene that prevents the synthesis of functional Eda protein are very well capable of inducing a protective immune response against virulent wild-type bacteria in the host animal. Such mutants are, first of all, safe when used as live attenuated vaccine strains. Moreover, they are capable of preventing colonisation of wild type strains.

15 Merely as an example, when such vaccines are administered to chickens of over two weeks of age they are capable of inducing immunity even to the level that neither the vaccine strain nor the challenge strain can be re-isolated from the cloaca after challenge.

This is indeed against all expectations, since it has been shown that *E. coli* Eda-negative
20 mutants do not colonise the mouse large intestine (Sweeney, N.J. et al., in Infect. & Immun. 64: 3504-3511 (1996)). This is even the case if the mice are previously streptomycin-treated. Such treatment leads to the eradication of all facultative bacteria from the intestines, so that all possible intestinal niches are in principle available. Nevertheless, Eda-negative mutants were shown, contrary to their wild-type counterparts, to be unable to colonise in mice. Thus, Eda-
25 negative mutants were also not expected to colonise the digestive tract of other mammals or poultry, so they consequently were not expected to come into sufficiently close contact with the immune system to trigger it anyway.

Therefore, one embodiment of the present invention relates to a live attenuated bacterium that
30 is not capable to express a functional Eda protein as a result of a mutation in the *eda* gene, for use in a vaccine.

Additionally, Eda-mutants as described in the invention show a very low pathogenicity, which makes them attractive as vaccine strains.

The *eda* gene, encoding the Eda protein plays a key role in what is known as the Entner-Doudoroff Pathway. Its gene product, the Eda-protein is an enzyme, also known as KDPG-aldolase. This enzyme is used in the metabolism of glucuronate, galacturonate and gluconate. Fraenkel, D.G. has described the Entner-Doudoroff pathway in e.g. *E.coli* and *Salmonella* at p. 142-150 in F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter and H.E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. 10 American Society for Microbiology, Washington D.C. (1987). Egan et al., have analysed the Entner-Doudoroff pathway in *E. coli* and they have analysed the sequence and localized promoters for the *edd-eda* operon (Egan et al., J. Bacteriology 174: 4638-4646 (1992)).

15 The sequence of the DNA encoding Eda of *Salmonella typhimurium* is depicted in SEQ ID NO: 1. The sequence of the Eda protein itself is given in SEQ ID NO: 2.

The fact that *eda* mutants are not expected to colonise the digestive tract explains why, although such mutants were known in the art, they have never been suggested to be potential 20 live attenuated vaccine candidates.

Due to its key position in carbohydrate metabolism, the *eda* gene and its gene product Eda are relatively widespread in the bacterial realm. The Eda protein is a highly conserved protein. It can be found in e.g. *Escherichia coli*, in *Salmonella* species, more specifically *Salmonella enterica* species, such as serotype Typhimurium, Enteritidis and Dublin and in *Yersinia* species 25 such as *Y. pestis*.

The mutation can be an insertion, a deletion, a substitution or a combination thereof, provided that the mutation leads to the failure to express a functional Eda protein. A functional Eda 30 protein is understood to be a protein having the regulating characteristics of the wild-type protein. Thus, an Eda protein that is defective in at least one of its functions is considered to be a non-functional Eda protein. More specifically, a non-functional Eda protein would not or to a

lesser extend, when compared to its wild-type counterpart, be able to mediate the synthesis of KDPG from pyruvate and glyceraldehyde-3-phosphate and vice versa. As a result, a strain having a non-functional Eda-protein would not or to a lesser extent, when compared to its wild-type counterpart, be able to utilise glucuronate, galacturonate and gluconate.

5

Live attenuated bacteria for use according to the invention can be obtained in several ways. One possible way of obtaining such bacteria is by means of classical methods such as the treatment of wild-type bacteria having the *eda* gene with mutagenic agents such as base analogues, treatment with ultraviolet light or temperature treatment.

10

Strains that do not produce a functional Eda protein can easily be picked up. Such mutants can not utilise glucuronate, galacturonate or gluconate, but they can utilise glucose and galactonate.

They can therefore, on the basis of these specific abilities, very easily be selected *in vitro*. A

15

detailed description of how to make such a selection has been described by Sweeney, N.J. et al., in Infect. & Immun. 64: 3504-3511 (1996).

The nature of a mutation if caused by classical mutation techniques is unknown. This may be a point mutation which may, although this is unlikely to happen, eventually revert to wild-type. In

20

order to avoid this small risk, transposon mutagenesis would be a good alternative.

Mutagenesis by transposon mutagenesis, is also a mutagenesis-technique well-known in the art. This is a mutation accomplished at a localised site in the chromosome. Transposon- insertions can not be targeted to a specific gene. It is however very easy to pick up *eda*- mutants since they do not grow *in vitro* without nutrient compensation for lack of Eda activity.

25

Therefore, they can easily be selected from a pool of randomly transposon-mutated bacteria.

A much more attractive way of making mutations, i.e. the introduction of a mutation at a predetermined site, rather deliberately than randomly, is offered by recombinant DNA- technology. Such a mutation may again be an insertion, a deletion, a replacement of one

30

nucleotide by another one or a combination thereof, with the only proviso that the mutated gene no longer encodes functional Eda. Such a mutation can e.g. be made by deletion of a number of base pairs. Even very small deletions such a stretches of 10 base pairs can already render

Eda non-functional. Even the deletion of one single base pair may already lead to a non-functional Eda, since as a result of such a mutation, the other base pairs are no longer in the correct reading frame. Each deletion or insertion of a number of base pairs indivisible by three causes such a frame shift. More preferably, a longer stretch is removed e.g. 100 base pairs.

5 Even more preferably, the whole *eda* gene is deleted.

It can easily be seen, that especially mutations introducing a stop-codon in the open reading frame, or mutations causing a frame-shift in the open reading frame are very suitable to obtain a strain which no longer encodes functional Eda.

All techniques for the construction of Eda-negative mutants are well-known standard

10 techniques. They relate to cloning of the Eda-gene, modification of the gene sequence by site-directed mutagenesis, restriction enzyme digestion followed by re-ligation or PCR-approaches and to subsequent replacement of the wild type *eda* gene with the mutant gene (allelic exchange or allelic replacement). Standard recombinant DNA techniques such as cloning the *eda* gene in a plasmid, digestion of the gene with a restriction enzyme, followed by
15 endonuclease treatment, re-ligation and homologous recombination in the host strain, are all known in the art and described i.a. in Maniatis/Sambrook (Sambrook, J. et al. Molecular cloning: a laboratory manual. ISBN 0-87969-309-6). Site-directed mutations can e.g. be made by means of in vitro site directed mutagenesis using the Transformer® kit sold by Clontech.
PCR-techniques are extensively described in (Dieffenbach & Dreksler; PCR primers, a
20 laboratory manual. ISBN 0-87969-447-3 and ISBN 0-87969-447-5).

The *eda* gene comprises not only the coding sequence encoding the Eda protein, but also regulatory sequences such as the promoter. The gene also comprises sites essential for correct translation of the Eda mRNA, such as the ribosome binding site.

25 Therefore, not only mutations in the coding regions but also mutations in those sequences essential for correct transcription and translation are considered to fall within the scope of the invention.

In a preferred embodiment, the invention relates to live attenuated bacteria of the genera

30 *Escherichia*, *Salmonella* and *Yersinia* for use in a vaccine.

In a more preferred form of the invention, the live attenuated bacterium according to the invention is selected from the group consisting of *S. enterica* serotype *typhimurium*, *enteritidis*, *choleraesuis*, *dublin*, *typhi*, *gallinarum*, *abortusovis*, *abortus-equii*, *pullorum*, *E. coli* or *Y. pestis*. These bacterial genera comprise a large number of species that are pathogenic to both
5 humans and a variety of different animals.

In an even more preferred form thereof, the live attenuated bacterium according to the invention is selected from the group consisting of *S. enterica* serotype *typhimurium*, *enteritidis*, *gallinarum*, *pullorum*, *E. coli* or *Y. pestis*.

10 Well-defined and deliberately made mutations involving the deletion of fragments of the *eda* gene or even the whole gene or the insertion of heterologous DNA-fragments or both, have the advantage, in comparison to classically induced mutations, that they will not revert to the wild-type situation.

15 Thus, in an even more preferred form, this embodiment of the invention refers to live attenuated bacteria in which the *eda* gene comprises an insertion and/or a deletion.

Given the large amount of vaccines given nowadays to both pets and farm animals, it is clear that combined administration of several vaccines would be desirable, if only for reasons of
20 decreased vaccination costs. It is therefore very attractive to use live attenuated bacteria as a recombinant carrier for heterologous genes, encoding antigens selected from other pathogenic micro-organisms or viruses. Administration of such a recombinant carrier has the advantage that immunity is induced against two or more diseases at the same time. The live attenuated bacteria for use in a vaccine, according to the present invention provide very suitable carriers
25 for heterologous genes, since the gene encoding the Eda protein can be used as an insertion site for such heterologous genes. The use of the *eda* gene as an insertion site has the advantage that at the same time the *eda* gene is inactivated and the newly introduced heterologous gene can be expressed (in concert with the homologous bacterial genes). The construction of such recombinant carriers can be done routinely, using standard molecular
30 biology techniques such as allelic exchange.

Therefore, another embodiment of the invention relates to live attenuated recombinant bacteria, preferably of the genera *Escherichia*, *Salmonella* and *Yersinia* that do not produce a functional Eda protein and in which a heterologous gene is inserted, for use in a vaccine. Such a heterologous gene can, as mentioned above, e.g. be a gene encoding an antigen selected from other pathogenic micro-organisms or viruses. Such genes can e.g. be derived from pathogenic herpesviruses (e.g. the genes encoding the structural proteins of herpesviruses), retroviruses (e.g. the gp160 envelope protein), adenoviruses and the like.

Also a heterologous gene can be obtained from pathogenic bacteria. As an example, genes encoding bacterial toxins such as *Actinobacillus pleuropneumoniae* toxins, *Clostridium* toxins, outer membrane proteins and the like are very suitable bacterial heterologous genes.

Another possibility is to insert a gene encoding a protein involved in triggering the immune system, such as an interleukin or an interferon, or another gene involved in immune-regulation.

Insertion of the heterologous gene in the *eda* gene is advantageous, since in that case there is no need to find a new suitable insertion site for the heterologous gene, and at the same time the *eda* gene is knocked out.

Thus, in a preferred form of this embodiment the heterologous gene is inserted in the *eda* gene. The heterologous gene can be inserted somewhere in the *eda* gene or it can be inserted at the site of the *eda* gene while this gene has been partially or completely deleted.

Because of their unexpected attenuated but immunogenic character *in vivo*, the bacteria for use in a vaccine, according to the invention are very suitable as a basis for live attenuated vaccines. Thus, still another embodiment of the invention relates to live attenuated vaccines for the protection of animals and humans against infection with a bacterium of which the wild type form comprises a *eda* gene.

Such vaccines comprise an immunogenically effective amount of a live attenuated bacterium for use in a vaccine, according to the invention or a live recombinant carrier bacterium according to the invention, and a pharmaceutically acceptable carrier.

Preferably, the vaccine comprises a live attenuated bacterium according to the invention, selected from the group of *Escherichia*, *Salmonella* and *Yersinia*.

Immunogenically effective means that the amount of live attenuated bacteria administered at vaccination is sufficient to induce in the host an effective immune response against virulent forms of the bacterium.

In addition to an immunogenically effective amount of the live attenuated bacterium described

5 above, a vaccine according to the present invention also contains a pharmaceutically acceptable carrier. Such a carrier may be as simple as water, but it may e.g. also comprise culture fluid in which the bacteria were cultured. Another suitable carrier is e.g. a solution of physiological salt concentration.

10 The useful dosage to be administered will vary depending on the age, weight and animal vaccinated, the mode of administration and the type of pathogen against which vaccination is sought.

The vaccine may comprise any dose of bacteria, sufficient to evoke an immune response.

15 Doses ranging between 10^3 and 10^{10} bacteria are e.g. very suitable doses.

Optionally, one or more compounds having adjuvant activity may be added to the vaccine.

Adjuvants are non-specific stimulators of the immune system. They enhance the immune response of the host to the vaccine. Examples of adjuvants known in the art are Freunds

20 Complete and Incomplete adjuvant, vitamin E, non-ionic block polymers, muramyldipeptides, ISCOMs (immune stimulating complexes, cf. for instance European Patent EP 109942), Saponins, mineral oil, vegetable oil, and Carbopol.

Adjuvants, specially suitable for mucosal application are e.g. the *E. coli* heat-labile toxin (LT) or *Cholera* toxin (CT).

25 Other suitable adjuvants are for example aluminium hydroxide, aluminium phosphate or aluminium oxide, oil-emulsions (e.g. of Bayol F (R) or Marcol 52 (R)), saponins or vitamin-E solubilisate.

Therefore, in a preferred form, the vaccines according to the present invention comprise an

30 adjuvant.

Other examples of pharmaceutically acceptable carriers or diluents useful in the present invention include stabilisers such as SPGA, carbohydrates (e.g. sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as albumin or casein, protein containing agents such as bovine serum or skimmed milk and buffers (e.g. phosphate buffer).

5 Especially when such stabilisers are added to the vaccine, the vaccine is very suitable for freeze-drying. Therefore, in a more preferred form, the vaccine is in a freeze-dried form.

For administration to animals or humans, the vaccine according to the present invention can be given inter alia intranasally, intradermally, subcutaneously, orally, by aerosol or intramuscularly.

10 For application to poultry, wing web and eye-drop administration are very suitable.

The skilled person would know how to administer a vaccine according to the invention, because the method would most likely not differ from the methods followed for vaccination with presently existing bacterial vaccines. A vaccine according to the invention, especially when it comprises bacteria belonging to the family of *E. coli*, *Salmonella* or *Yersinia* would preferably be given

15 orally.

Still another embodiment relates to the use of a bacterium or a recombinant bacterium according to the invention for the manufacture of a vaccine for the protection of animals and humans against infection with a wild type bacterium or the pathogenic effects of infection.

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Still another embodiment of the invention relates to methods for the preparation of a vaccine according to the invention. Such methods comprise the admixing of a live attenuated bacterium according to the invention or a live recombinant carrier bacterium according to the invention, and a pharmaceutically acceptable carrier.

25

EXAMPLES**Example 1****Construction of Eda-negative mutant.**

The *eda* deletions were made using a modified Wanner method (PNAS June 6, 2000).

5 97(12):6640-45). Primers to the 5' and 3' *eda* flanking sequences of *S. enteritidis* SE5609 were designed with *PstI* sites (*PstI* *eda* 5': cta gct gca ggt gct aag cgg taa tct ggg and *PstI* *eda* 3': cta gct gca gaa gag att gct cgt cat gtg g) and the PCR product was cloned into pBluescript SKII+ (pBSeda). *BglII* containing primers, to the *eda* promoter region (ctag aga tct ctcgcctgattacta gtgtg) and the 3' end (ctag agatct aag ccg ttaaatgcccgttgg) were used to reverse PCR amplify
10 the flanking and vector sequences from pBSeda. *BglII* digestion and subsequent ligation created an *eda* deletion clone called pBSedaΔ. A 1.2 kb *BamHI* digested chloramphenicol resistant gene was inserted into the *BglII* site of pBSedaΔ to produce pBSedaΔcam.
15 pBSedaΔcam was digested with *PstI* and the insert was separated from vector sequences and used as the template for PCR. Eight 100 µl reactions were pooled, 5 µl was checked on a gel, and the linear PCR product was ethanol precipitated and resuspended in 2-4 µl of water.

S. enteritidis SE5609 cells were electroporated with the temperature sensitive plasmid pKD46. When these cells are grown at 30°C in the presence of arabinose the plasmid expresses the lambda Red recombinase. The cells ($A_{600}=0.6$) were made competent for electroporation by
20 centrifugation and washing 3-4X with cold 10% glycerol. The linear PCR product was then electroporated into the competent cells. The recombinase acts to replace the wild type *eda* gene with the deleted *eda* gene containing the chloramphenicol cassette. SE5609 clones containing the deleted *eda* gene containing the chloramphenicol cassette were selected by overnight growth at 37°C on Luria agar plates containing chloramphenicol (30 µg/ml), which
25 also resulted in loss of the temperature sensitive pKD46 plasmid.

A different set of flanking *eda* 5' (ctagctgca gcc tca tat tcc gga cct gag c) and *eda* 3' (cta gct gca ggt gac ggt aaa agg cta atg cg) primers were used to verify one of the SE5609-*eda*⁽⁺⁾ *eda* mutants. An amplified fragment containing the wild type SE5609 *eda* gene produced a 927bp
30 band as expected, while the *eda* deletion/chloramphenicol mutant gave the expected 1677bp product. 1.8mM MgCl₂, with Finnzyme DyNAzyme II polymerase was used in the reaction. Cycling conditions were 1X 94°C 4min; 30X 94°C 15sec, 55°C 30sec, 72°C 130sec; 1X 72°C

7min. In addition, the same SE5609-eda⁽⁺⁾ eda mutant was also confirmed by its inability to grow in liquid M9 minimal medium containing 0.2% (w/w) gluconate as the sole carbon source.

5 Example 2

Safety, vaccination- and challenge-tests.

Experimental design

To test both safety and efficacy, chickens were orally inoculated at 6 and 14 weeks of age with respectively 1.1x10⁸ CFU and 4.1x10⁸ CFU of SE5609-eda⁽⁺⁾.

10 Safety was assessed by clinical observation after vaccination. Also, cloaca swabs were taken at days 7 and 14 after each vaccination to determine the presence of the vaccine strain in the intestinal tract. Swabs were used to inoculate Brilliant Green Agars (BGA) directly and after enrichment in Rappaport Vassiliades Broth.

To test for efficacy, the vaccinated chickens and unvaccinated controls received an oral

15 challenge infection with 1.3x10⁸ CFU of a naladixic acid resistant wild-type S.e. strain at 16 weeks of age. Cloaca swabs were taken 3, 7 and 14 days after challenge to determine the rate of colonization by the challenge strain. Swabs were inoculated on BGA containing naladixic acid (BGAnal) directly and also after incubation in an enrichment medium (buffered peptone water containing nal).

20

Animals

Commercial laying hens were obtained from a Salmonella free flock.

Results

25 No clinical abnormalities were observed after both oral vaccinations.

First of all, the SE5609-eda⁽⁺⁾ strain was not cultured from cloacal swabs of the vaccinated animals on days 7 and 14, indicating that the strain was impaired in its ability to colonize the digestive tract of chickens.

Moreover, as shown in Table 1, vaccination with SE5609-eda⁽⁺⁾ resulted in a very significant

30 reduction in colonization of the digestive tract by the challenge strain.

Table 1 Reisolation of the challenge strain from cloaca swabs

	S.e. (nal ^r) positive	
Day post challenge	SE5609-eda	Control
3	1/15 ^a	11/15
7	1/15 ^a	9/14
14	0/15	2/14

^a: significantly different from control (p<0.01, Fisher exact test)

Conclusion: a vaccine based upon a *Salmonella enteritidis* eda⁽⁺⁾ strain which is a wild-type strain having eda⁽⁺⁾ as a single attenuation is safe. Moreover, vaccination with this *Salmonella enteritidis* eda⁽⁺⁾ strain resulted in complete prevention of colonization of the digestive tract by both the vaccine strain and the challenge strain.

Claims

- 1) Live attenuated bacterium of the genus *Escherichia*, *Salmonella* or *Yersinia*, said bacterium not being capable to express a functional Eda protein as a result of a mutation in the *eda* gene,
5 for use in a vaccine.
- 2) Live attenuated bacterium for use according to claim 1, wherein said bacterium is selected from the group consisting of *E. coli*, *Y. pestis*, *S. enterica* serotype *typhimurium*, *enteritidis*, *choleraesuis*, *dublin*, *typhi*, *gallinarum*, *abortusovi*, *abortus-equi*, *pullorum*.
- 10 3) Live attenuated bacterium for use according to claim 1 or 2, characterised in that the mutation comprises an insertion and/or a deletion.
- 15 4) Live attenuated bacterium for use according to claims 1-3, characterised in that said bacterium carries a heterologous gene.
- 5) Live attenuated bacterium for use according to claim 4, characterised in that said heterologous gene is inserted in the *eda* gene
- 20 6) Live attenuated vaccine for the protection of animals or humans against infection with a pathogenic bacterium or the pathogenic effects thereof, characterised in that said vaccine comprises a bacterium as defined in claims 1-5 and a pharmaceutically acceptable carrier.
- 7) Live attenuated vaccine according to claim 6, characterised in that it comprises an adjuvant.
- 25 8) Live attenuated vaccine according to claim 6 or 7, characterised in that it is in a freeze-dried form.
- 9) Use of a live attenuated bacterium as defined in claims 1-5 for the manufacture of a vaccine
30 for the protection of animals against infection with a pathogenic bacterium or the pathogenic effects of infection.

10) Method for the preparation of a vaccine according to claims 6-8, characterised in that said method comprises the admixing of a live attenuated bacterium as defined in claims 1-5 and a pharmaceutically acceptable carrier.

SEQUENCE LISTING

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Glu Gly Ala Lys Gln
210

INTERNATIONAL SEARCH REPORT

Inten application No
PCT/US2004/027897

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 A61K39/02 C12N1/20 A61P31/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, CHEM ABS Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 1 074 266 A (AKZO NOBEL N.V; THE BOARD OF GOVERNORS FOR HIGHER EDUCATION, STATE OF) 7 February 2001 (2001-02-07) page 1, paragraph 1 page 1, paragraph 5 - page 2, paragraph 10 claims	1-10
Y	SWEENEY N J ET AL: "ESCHERICHIA COLI F-18 AND E. COLI K-12 EDA MUTANTS DO NOT COLONIZE THE STREPTOMYCIN-TREATED MOUSE LARGE INTESTINE" INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON, US, vol. 64, no. 9, September 1996 (1996-09), pages 3504-3511, XP000905643 ISSN: 0019-9567 cited in the application the whole document	1-10
	-/-	

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the International filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the International filing date but later than the priority date claimed

T later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the International search

Date of mailing of the International search report

26 January 2005

08/02/2005

Name and mailing address of the ISA

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Rankin, R

INTERNATIONAL SEARCH REPORT

Inte	Application No
	PCT/US2004/027897

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATSENKO KIRILL A ET AL: "One-step inactivation of chromosomal genes in <i>Escherichia coli</i> K-12 using PCR products" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, US, vol. 97, no. 12, 6 June 2000 (2000-06-06), pages 6640-6645, XP002210218 ISSN: 0027-8424 cited in the application abstract -----	1-10
A	ALLEN J H ET AL: "A functional cra gene is required for <i>Salmonella enterica</i> serovar Typhimurium virulence in BALB/c mice" INFECTATION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, US, vol. 68, no. 6, June 2000 (2000-06), pages 3772-3775, XP002229250 ISSN: 0019-9567 the whole document -----	1-10
A	RAMSEIER T M: "CRA AND THE CONTROL OF CARBON FLUX VIA METABOLIC PATHWAYS" RESEARCH IN MICROBIOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 147, no. 6/7, 1996, pages 489-493, XP001134939 ISSN: 0923-2508 the whole document -----	1-10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2004/027897

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
 - a. type of material
 - a sequence listing
 - table(s) related to the sequence listing
 - b. format of material
 - in written format
 - in computer readable form
 - c. time of filing/furnishing
 - contained in the international application as filed
 - filed together with the international application in computer readable form
 - furnished subsequently to this Authority for the purpose of search
2. In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte	Application No
	PCT/US2004/027897

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 1074266	A 07-02-2001	US 6764687 B1 AU 3935300 A BR 0002602 A CA 2308691 A1 EP 1074266 A2 HU 0002228 A2 JP 2001039890 A MX PA00005734 A NZ 505018 A ZA 200002615 A	20-07-2004 14-12-2000 02-01-2001 09-12-2000 07-02-2001 29-06-2002 13-02-2001 20-08-2002 27-04-2001 08-12-2000